Metabolites of a Tobacco-specific Nitrosamine, 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), in the Urine of Smokeless Tobacco Users: Relationship between Urinary Biomarkers and Oral Leukoplakia[1].

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Abstract

Two metabolites of the carcinogenic tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone were quantified in the urine of smokeless tobacco users. The metabolites are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid (NNAL-Gluc). The study population consisted of 47 male nonsmokers, of whom 23 were snuff dippers, 13 were tobacco chewers, 3 were users of both products, and 8 were nonusers. The levels of NNAL-Gluc in urine ranged from 0.14–30.3 pmol/mg creatinine with a mean ± SD of 3.47 ± 5.86, whereas the levels of NNAL ranged from 0.02–8.73 pmol/mg creatinine with a mean ± SD of 0.92 ± 1.59. The mean levels of NNAL-Gluc and NNAL were not significantly different from those measured in a previous study of smokers. The levels of NNAL-Gluc were significantly higher in snuff dippers than in tobacco chewers. The ratio of NNAL-Gluc:NNAL was higher in snuff dippers than in tobacco chewers or smokers. There was no indication of two phenotypes of the NNAL-Gluc:NNAL ratio in smokeless tobacco users, in contrast to previous observations in smokers. Of the 39 smokeless tobacco users in this study, 16 presented with oral leukoplakia. When the total levels of NNAL-Gluc, NNAL, or NNAL-Gluc + NNAL were divided into tertiles, there was a significant association between the presence of leukoplakia and increasing levels of these metabolites; a similar relationship was found between urinary cotinine and leukoplakia. The results of this study demonstrate that there is significant uptake of carcinogenic nitrosamines in smokeless tobacco users, and that such products are not harmless alternatives to cigarettes. Moreover, the urinary biomarkers NNAL-Gluc, NNAL, and cotinine were associated with the presence of leukoplakia, which provides biochemical support for the role of smokeless tobacco products as a cause of oral leukoplakia.

Introduction

Approximately 5.3 million adults, predominantly males, were users of smokeless tobacco in the U.S. in 1991. Use was highest in young males, American Indians/Alaskan natives, people living in the south or rural areas of the U.S., and those of low socioeconomic status (1, 2). There are two main types of smokeless tobacco: snuff and chewing tobacco (3). Snuff is a finely ground tobacco product that is placed in the gingival buccal area, a practice known as "snuff dipping". Snuff can be moist, dry, or in "tea bag" form. Chewing tobacco consists of tobacco in the form of loose leaf, plug, or twist. Since the 1970s, snuff dipping has enjoyed considerable popularity in the U.S. Smokeless tobacco use is widely practiced in many other parts of the world, most notably Scandinavia, India, and other parts of Asia (3).

Smokeless tobacco use, particularly snuff dipping, is an accepted cause of oral leukoplakia and oral cancer (4–9). Oral leukoplakia, which is considered to be a precursor lesion to oral cancer, is a white patch or plaque in the oral cavity that cannot be characterized clinically or pathologically as any other disease (7). The most abundant strong carcinogens in smokeless tobacco are the tobacco-specific nitrosamines, which form during the curing and fermentation of tobacco (10). Substantial amounts of other nitrosamines are also present (11). Among the tobacco-specific nitrosamines present in smokeless tobacco products, NNK[3] and NNN are the strongest carcinogens (12). The levels of NNK and NNN in currently available U.S. smokeless tobacco products are typically 1–10 ppm (13, 14). Tumors were induced in the rat oral cavity and lung upon chronic oral swabbing of a mixture of NNK and NNN in the same relative concentrations as they occur in snuff (15); oral swabbing of NNK alone induced only lung tumors (16). NNK and NNN in combination are likely to play a significant role in the induction of oral cancer in humans by smokeless tobacco (17–19).

Tobacco-specific nitrosamines have been detected in the

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[3] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrosonornicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NNAL-Gluc, [4-(methylnitrosamino)-1-(3-pyridyl)but-1-yl]-β-*O*-D-glucosiduronic acid.

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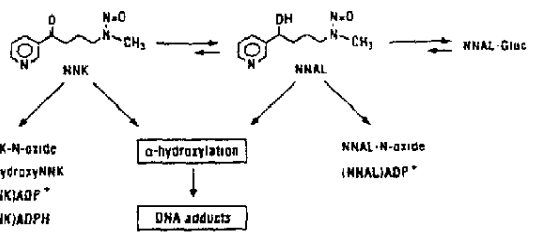

Fig. 1. Overview of NNK metabolism. NNAL is formed by carbonyl reduction and undergoes conjugation to NNAL-Gluc (see Refs. 36 and 37). The metabolic activation of NNK and NNAL proceeds by α-hydroxylation, leading to a complex mixture of metabolites, some of which form DNA adducts (see Ref. 12). Other metabolites of NNK and NNAL result from pyridine oxidation (NNK-*N*-oxide, NNAL-*N*-oxide, and 6-hydroxyNNK) or the substitution of nicotinamide in $NADP^+$ or NADPH by NNK or NNAL.

saliva of smokeless tobacco users in several studies (20–28). These studies demonstrated that the nitrosamines are extracted from the tobacco during chewing or snuff dipping. Hemoglobin adducts of NNN and NNK have been detected in the red blood cells of smokeless tobacco users, indicating that these carcinogens undergo metabolic activation in humans (29–31). However, relatively little information is available on the urinary levels of nitrosamine metabolites in smokeless tobacco users. We have developed methodology to quantify two urinary metabolites of NNK in humans: NNAL and NNAL-Gluc (Fig. 1; Refs. 32 and 33). NNAL, like NNK, is a potent carcinogen in rats and mice (34, 35). NNAL-Gluc is believed to be a detoxification product of NNK (36, 37). The ratio of NNAL-Gluc: NNAL in urine has been proposed as a potential marker of NNK detoxification (33). In smokers, the levels of urinary NNAL + NNAL-Gluc correlate with those of urinary cotinine and give a good indication of NNK uptake (32, 33). The levels of NNAL and NNAL-Gluc in the urine of Sudanese snuff dippers exposed to extraordinary amounts of NNK have been quantified (31).

In this study, we analyzed the urine of a group of snuff dippers and tobacco chewers from Ohio for NNAL and NNAL-Gluc. Our major goal was to establish the levels of urinary NNAL and NNAL-Gluc excretion as well as the NNAL-Gluc: NNAL ratios in typical smokeless tobacco users and to compare these data to those that we have obtained previously in smokers. We also investigated the relationship between the levels of urinary NNK metabolites and cotinine and the incidence of oral leukoplakia.

Materials and Methods

Subjects. This study was approved by the Ohio State University Biomedical Sciences Review Committee. All subjects signed consent forms and agreed to donate urine, have an oral cavity exam, and answer questions regarding tobacco use, diet, and medical history. Male smokeless tobacco users and controls were recruited through advertisements at the Ohio State University campus and the surrounding area. All subjects were nonsmokers. Tobacco chewers and snuff dippers had been regular users of smokeless tobacco for a minimum of 1 year. Each enrollee received an oral cavity examination.

Collection of Urine. Each participant collected 24-h urine samples in 3.5 liter amber specimen containers to which 10 ml of 20% ammonium sulfamate in 3.6 N H_2SO_4 had been added (33). Urine samples were stored at room temperature during the 24-h collection period and then were aliquoted into two 4-oz amber bottles and one 8-oz amber bottle and were stored at −80°C. Batches of samples were shipped on dry ice to the American Health Foundation for analysis.

Analysis of Urine. NNAL and NNAL-Gluc were determined by gas chromatography with detection by a nitrosamine-selective chemiluminescence detector, the Thermal Energy Analyzer (33). Cotinine was determined by RIA using antisera produced in rabbits (38). Creatinine was measured with a Kodak Ektachem 500 clinical chemistry analyzer.

Oral Cavity Exam. An oral pathologist (R. G.) conducted an oral cavity examination on each study participant. The exam included evaluation of the gross appearance of the lips, oral mucosa, palate, tongue, mouth floor, oropharynx, and teeth. General oral hygiene and the presence of gingivitis and leukoplakia were also appraised.

Statistical Methods. Comparisons of NNAL, NNAL-Gluc, the NNAL-Gluc:NNAL ratio, and cotinine among the groups were carried out by ANOVA on log-transformed data. The relationship of urinary biomarkers to the prevalence of leukoplakia was quantified using logistic regression analysis. Statistical significance was set at the 0.05 level, with odds ratios and 95% confidence intervals presented (Table 3). Tests for trends were performed by the methods of Mantel and Haenszel, using the one degree of freedom χ^2 test (39).

Results

The study population consisted of nonsmoking males; 13% of the participants reported being ex-smokers. The average ages of the snuff dippers and chewers were 27 and 25 years, respectively. Ninety-two percent of the subjects were Caucasian. Eighty-nine percent of the snuff dippers dipped at least once a day, and 50% reported dipping over four times a day. Among the chewers, 50% reported chewing 3–6 times per week, and the remaining 50% reported chewing more than once a day.

The levels of urinary NNAL-Gluc and NNAL as well as the NNAL-Gluc:NNAL ratios in 39 smokeless tobacco users are summarized in Table 1. There was a wide range of NNAL and NNAL-Gluc values, which varied more than 400-fold. There was over a 20-fold variation in the NNAL-Gluc:NNAL ratios. When the highest NNAL-Gluc:NNAL ratio (36) was excluded, there was a 4.6-fold variation in the NNAL-Gluc: NNAL ratios.

Urinary NNAL and NNAL-Gluc were also analyzed in eight Caucasian male control subjects (average age, 31 years) who did not use tobacco products of any type and had urinary cotinine levels of less than 50 ng/ml. Free NNAL was not detected in these subjects. NNAL-Gluc was detected in three subjects, ranging from 0.07–0.12 pmol/mg creatinine.

The mean levels of NNAL-Gluc, NNAL, and cotinine as well as the NNAL-Gluc:NNAL ratios in smokeless tobacco users, snuff dippers, and chewers are compared to those in smokers in Table 2. The data for 61 smokers are from a previous study. The levels of NNAL-Gluc and cotinine were significantly higher in snuff dippers and smokers than in chewers. The levels of NNAL-Gluc, NNAL, and cotinine in all smokeless tobacco users were not significantly different from those in smokers.

The ratio of NNAL-Gluc:NNAL was significantly higher in snuff dippers than in smokers ($P = 0.04$). It was also higher in snuff dippers than in chewers, but this difference was not significant ($P = 0.08$). There was no significant difference in the NNAL-Gluc:NNAL ratio between chewers and smokers or between smokeless tobacco users and smokers. Frequency plots

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Table 1 Urinary excretion of NNAL-Gluc, NNAL, and cotinine in 39 smokeless tobacco users

Subject	Tobacco type[a]	NNAL-Gluc (pmol/mg creatinine)	NNAL (pmol/mg creatinine)	NNAL-Gluc/NNAL ratio	Cotinine (nmol/mg creatinine)
1	D	1.58	0.45	3.5	9.37
2	D	1.51	0.26	5.4	3.80
3	D	1.28	0.37	3.4	0.66
4	D	30.3	8.73	3.5	9.30
5	D	0.75	0.20	3.7	9.47
6	D	0.73	0.02	36	0.48
7	D	1.07	0.49	2.2	20.0
8	D	0.64	0.23	2.8	4.80
9	D	1.12	0.27	4.2	6.50
10	D	1.81	0.52	3.5	7.52
11	D	0.80	0.20	4.1	1.10
12	D	1.78	0.80	2.2	34.3
13	D	23.0	5.71	4.1	23.3
14	D	2.46	0.51	4.9	7.60
15	D	4.66	1.09	4.3	38.1
16	D	1.73	0.48	3.6	6.34
17	D	6.77	1.23	5.5	17.1
18	D	5.16	1.19	4.3	29.3
19	D	4.45	0.94	4.8	38.9
20	D	6.43	0.87	7.4	9.00
21	D	0.97	0.24	4.0	3.30
22	D	5.57	1.43	3.9	19.0
23	D	2.79	1.46	1.9	13.5
24	C	2.38	0.88	2.7	11.1
25	C	1.48	0.57	2.6	6.20
26	C	5.92	2.09	2.8	38.9
27	C	0.38	0.08	4.8	0.33
28	C	0.86	0.52	1.6	2.45
29	C	0.54	0.19	2.9	1.78
30	C	0.74	0.31	2.4	3.65
31	C	1.04	0.27	3.8	2.51
32	C	0.14	0.07	1.9	1.05
33	C	0.33	0.10	3.4	1.11
34	C	0.26	0.06	4.2	1.68
35	C	0.48	0.13	3.6	3.93
36	C	5.82	1.11	5.3	19.0
37	C/D	2.57	0.67	3.8	18.2
38	C/D	3.30	0.61	5.4	13.9
39	C/D	1.54	0.56	2.8	17.5
Mean ± S.D		3.47 ± 5.86	0.92 ± 1.59	4.5 ± 5.3	11.7 ± 11.4
Range		0.14–30.3	0.02–8.73	1.6–36	0.33–38.9

[a] C, chewers (chewing tobacco); D, dippers (snuff).

of the NNAL-Gluc:NNAL ratios for dippers and chewers are illustrated in Fig. 2, *A* and *B*. These can be compared to a frequency plot of the NNAL-Gluc:NNAL ratios for smokers, illustrated in Fig. 2*C*. Two phenotypes of the NNAL-Gluc: NNAL ratio were observed in smokers but were not evident in either dippers or chewers.

Sixteen of the 39 subjects presented with oral leukoplakia. Of these, 11 were snuff dippers, 2 were chewers, and 3 reported using both products. The relationship between the levels of urinary NNAL-Gluc, NNAL, NNAL-Gluc + NNAL, and cotinine and the incidence of oral leukoplakia is summarized in Table 3. The levels of these metabolites were divided into tertiles. The numbers of subjects with leukoplakia present increased with increasing levels of the metabolites in the tertiles, whereas the reverse was observed in the subjects with leukoplakia absent. These trends were significant for NNAL-Gluc, NNAL, NNAL-Gluc + NNAL, and cotinine. Although the number of subjects is small, the positive trend tests support the presence of a dose response.

Discussion

This is the first report describing the levels of NNAL and NNAL-Gluc in the urine of U.S. smokeless tobacco users. Because NNAL and NNAL-Gluc seem to be quantitatively significant metabolites of NNK, they provide an indication of systemic exposure to this carcinogen. The results demonstrate that the levels of NNAL and NNAL-Gluc in the urine of smokeless tobacco users are similar to those in smokers. Urinary cotinine levels were also similar in smokeless tobacco users and smokers, suggesting similar uptake of nicotine in these two groups, as noted previously (40, 41). These results indicate that systemic NNK exposure is similar in smokeless tobacco users and smokers, although the tissue distribution of NNK would likely differ. Nevertheless, the results emphasize the potential hazards of smokeless tobacco use because NNK is a potent carcinogen.

Based on a mean creatinine excretion of 2.1g/24 h, mean NNAL + NNAL-Gluc in urine can be estimated as approximately 9 nmol/24 h (2 μg/24 h) in smokeless tobacco users. This corresponds to the minimum daily uptake of NNK because the percentage conversion of NNK to NNAL + NNAL-Gluc is not known.

A potentially important finding of this study was the relationship between the level of NNAL, NNAL-Gluc, or cotinine in urine and the presence of oral leukoplakia. Urinary biomarkers in the highest tertiles resulted in elevated odds ratios reflecting a 23- to 35-fold increase in the relative risk of leukoplakia. Additionally, these data reflect a significant dose response in the relative risk of leukoplakia at increasing levels of each urinary metabolite. Although our study is small, it indicates that NNAL, NNAL-Gluc, and cotinine levels in urine may be useful biomarkers for leukoplakia development. Previous studies have demonstrated a relationship between the dose of smokeless tobacco, especially oral snuff products, and the incidence of leukoplakia. In one large study, the prevalence of oral leukoplakia among smokeless tobacco users increased with the hours used per day and decreased with the time elapsed since last use (9, 42). The relationship that we have observed is consistent with these results, if one considers that NNAL, NNAL-Gluc, and cotinine are biochemical markers of smokeless tobacco dose. Only one previous study has attempted to assess the relationship between nicotine metabolites in urine and oral leukoplakia in smokeless tobacco users; significant associations were not detected (43). The relationship between the NNK metabolites NNAL and NNAL-Gluc and the prevalence of oral leukoplakia is biologically plausible because a mixture of NNK and the related tobacco-specific nitrosamine NNN induced oral tumors when applied to the mucosa of the rat oral cavity (15).

The ratio of NNAL-Gluc:NNAL is a potential biomarker of NNK detoxification because NNAL-Gluc is a likely detoxification product of NNK, whereas NNAL is a carcinogen with activity similar to that of NNK (34–37). In a previous study, we noted two phenotypes of this ratio in smokers, as illustrated in Fig. 2*C* (33). The lower phenotype ranged from 0–5, whereas the higher phenotype ranged from 6–11. The latter group would presumably be at lower risk. No indication of the two phenotypes was seen in the present study. The distribution of ratios was normal in both snuff dippers and chewers, and there was no suggestion of a high glucuronidation phenotype. The study subjects in these two investigations were quite different. The

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Table 2 Comparison of urinary NNAL and NNAL-Gluc in smokeless tobacco users and smokers[a]

	NNAL-Gluc (pmol/mg creatinine)		NNAL (pmol/mg creatinine)		NNAL-Gluc/NNAL Ratio		Cotinine[b] (nmol/mg creatinine)	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Smokeless tobacco users (n = 39)	3.47 ± 5.86	0.14–30.3	0.92 ± 1.59	0.02–8.73	4.5 ± 5.3	1.6–36	11.7 ± 11.4	0.33–38.9
Snuff dippers (n = 23)	4.67 ± 7.28[c]	0.64–30.3	1.20 ± 2.00	0.02–8.73	5.36 ± 6.79[d]	1.9–36	13.7 ± 11.9[e]	0.48–38.9
Chewers (n = 13)	1.57 ± 2.00	0.07–5.92	0.49 ± 0.58	0.06–2.09	3.23 ± 1.09	1.3–5.3	7.21 ± 10.8	0.33–38.9
Smokers (n = 61)	2.81 ± 2.92[c]	0.16–19.0	0.95 ± 1.15[c]	0.08–4.89	3.7 ± 2.3	0.7–10.8	10.05 ± 8.34[e]	0.74–38.5

[a] Data for smokers are from Ref. 33
[b] Determined by RIA (except in smokers, which were analyzed by gas chromatography-mass spectroscopy).
[c] Significantly greater than chewers, $P < 0.01$
[d] Significantly greater than smokers, $P = 0.04$.
[e] Significantly greater than chewers, $P \leq 0.05$.

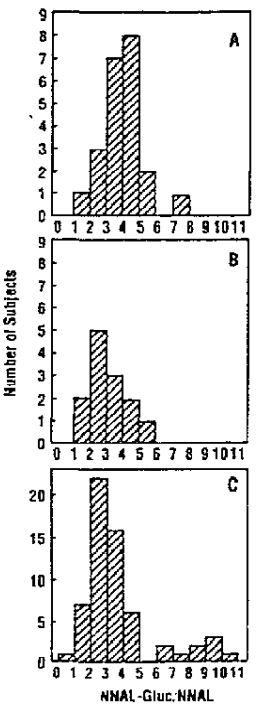

Fig. 2 Frequency plots of the NNAL-Gluc:NNAL ratios in *A*, snuff-dippers; *B*, tobacco chewers, and *C*, smokers. The data in *C* are from Ref. 33. In *A*, one subject with a ratio of 36 is not shown.

smokers were a biracial group from Mount Vernon, New York, consisting of 34 Blacks and 27 Caucasians, approximately 50% male, 50% female. The smokeless tobacco users were predominantly Caucasian males from Ohio. In spite of these differences, the data suggest that the high glucuronidation phenotype, which was not observed in the present study of nonsmokers, may result from the induction of UDP-glucuronosyl transferase activity by cigarette smoke. A number of previous studies have noted increased levels of drug glucuronidation in heavy smokers, whereas other investigations have not detected such an effect (44).

The mean ratio of NNAL-Gluc:NNAL was higher in snuff dippers than in chewers and smokers. The origin of this difference is unknown, but it could be influenced by a constituent of

Table 3 Relationship of levels of urinary biomarkers to presence of oral leukoplakia

	Number (%) of subjects with				
	Leukoplakia present (n = 16)	Leukoplakia absent (n = 23)	OR[a] (95% CI)		P-trend
NNAL-Gluc (pmol/mg creatinine)					
≤1.0	2 (12.5)	11 (47.8)			
1.1 to 2.0	4 (25.0)	7 (30.4)	3.7	(0.41–34)	
>2.0	10 (62.5)	5 (21.7)	23	(2.5–210)	0.006
NNAL (pmol/mg creatinine)					
≤0.45	3 (18.8)	14 (60.9)			
0.46 to 0.90	6 (37.5)	6 (26.1)	13	(1.5–116)	
>0.90	7 (43.8)	3 (13.0)	26	(2.7–254)	0.008
NNAL-Gluc+NNAL (pmol/mg creatinine)					
≤1.0	1 (6.3)	9 (39.1)			
1.1 to 3.0	6 (37.5)	9 (39.1)	10	(0.8–129)	
>3.0	9 (56.3)	5 (21.7)	35	(2.6–487)	0.010
Cotinine (nmol/mg creatinine)					
<4	1 (6.3)	13 (56.5)			
4 to 12	5 (31.3)	6 (26.1)	9.8	(0.9–113)	
>12	10 (62.5)	4 (17.4)	30	(2.9–318)	0.001

[a] Age adjusted. CI, confidence interval.

snuff. Some brands of snuff contain large amounts of additives such as methyl and ethyl salicylate, which are associated with the "wintergreen flavor" of snuff (45). In one study, salicylate administration selectively enhanced renal glucuronide conjugation of phenols in rats (46).

In summary, this study has demonstrated that the levels of NNK metabolites NNAL-Gluc and NNAL in the urine of smokeless tobacco users are similar to those in smokers. Therefore, smokeless tobacco cannot be considered a harmless alternative to smoking. Moreover, the urinary levels of NNAL, NNAL-Gluc, and cotinine were significantly associated with the presence of oral leukoplakia in smokeless tobacco users, strengthening the connection between the use of these products and the development of precancerous lesions of the oral cavity.

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